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CLONING AND PRODUCTION OF HUMAN ACETYLCHOLINESTERASE

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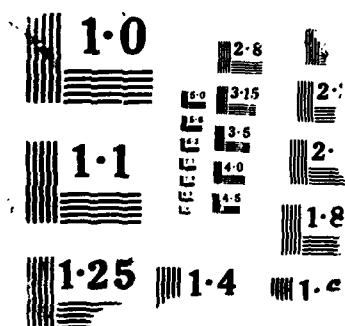
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HUMAN ACETYLCHOLINESTERASE

ANNUAL SUMMARY REPORT

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) <u>SUMMARY</u> The goal of this work is to clone by recombinant technology the gene for acetylcholinesterase (EC 3.1.1.7) from a human source for the purpose of producing large amounts of pure enzyme from a single gene. To this end we have screened five different established neuroblastoma cell lines for the production of enzyme. From the highest producer we have translated total messenger RNA in a rabbit reticulocyte system. We are currently preparing monoclonal antibody		

directed against acetylcholinesterase to isolate specific mRNA from a polyribosomal preparation from neuroblastoma SK-N-SH cells. *Requires:*

Growth, differentiation, biosynthesis 



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SUMMARY

The goal of this work is to clone by recombinant technology the gene for acetylcholinesterase (EC 3.1.1.7) from a human source for the purpose of producing large amounts of pure enzyme from a single gene. To this end we have screened five different established neuroblastoma cell lines for the production of enzyme. From the highest producer we have translated total messenger RNA in a rabbit reticulocyte system. We are currently preparing monoclonal antibody directed against acetylcholinesterase to isolate specific mRNA from a polyribosomal preparation from neuroblastoma SK-N-SH cells.

We have maintained five established cell lines of neuroblastoma in this laboratory. They are designated LAN-1, LAN-2, SK-N-SH, SK-N-MC, and IMR-32. A characteristic of neuroblastoma is their ability to synthesize proteins representative of a neuronal type cell. These cells have been classified as adrenergic, cholinergic, or mixed type based upon the presence of activity of neurotransmitter synthesizing enzymes. Accordingly, SK-N-SH, IMR-32, and LAN-1 are adrenergic since they contain, in increasing amounts, tyrosine hydroxylase activity, the rate limiting enzyme in catecholamine biosynthesis. LAN-2 is of the mixed type since it contains all of the activities of both adrenergic and cholinergic enzymes. All four of the above cell lines also exhibit activity of enzymes leading to biosynthesis of norepinephrine e.g. aromatic-L-amino decarboxylase and dopamine- β -hydroxylase. SK-N-MC is the only line in our laboratory which has the capacity to synthesize acetylcholine due to its choline acetyl transferase function.

Acetylcholinesterase assay:

To assay for acetylcholinesterase activity, medium was removed, and cells were processed according to Massoulie et al. (1982). Cells were washed with phosphate buffered saline and homogenized in 10 mM Tris/50 mM $MgCl_2$ /1% triton-X100/1.0M NaCl pH 7.0 buffer. Nuclei and cell debris were centrifuged at low speed and the supernatant collected. Activity was measured using a modification of the method of Rotondo et al. (1979) with [^{14}C]-acetylcholine as a substrate. Aliquots of cell extract were incubated with 1.2 mM acetylcholine in 100 mM sodium phosphate buffer pH7. The reaction was terminated with 50 mM glycine-HCl buffer, pH 2.5. The radioactive product of the reaction, [^{14}C] acetate, was extracted into the scintillation cocktail and counted directly. Under conditions of the reaction, the assay is linear with time and enzyme concentration.

Acetylcholinesterase activity in five cell lines:

Table I shows a comparison of acetylcholinesterase activity in the five different neuroblastoma cell lines. As can be seen S-K-N-SH cells consistently exhibited the higher acetylcholinesterase activity. To test the specificity of this reaction, two esterase inhibitors were used at appropriate concentrations. BW284c51 inhibits true acetylcholinesterase selectively, whereas IsoOMPA is an inhibitor of butyryl- or pseudocholinesterase. As can be seen from Table II, 95% of the activity of SK-N-SH neuroblastoma is true acetylcholinesterase with no detectable pseudocholinesterase activity. Therefore SK-N-SH neuroblastoma was chosen as the best candidate for the isolation of acetylcholinesterase messenger RNA.

Acetylcholinesterase activity has been induced in mouse neuroblastoma cells grown in serum free (1% serum) media (Harkins et al., 1972), but human neuroblastoma cells have not shown this response in our laboratory. Although we have seen a slight induction of esterase activity by supplementing medium with acetylcholine in the 1-5 mM range, this increase in activity was not dramatic enough to pursue in our scheme isolate specific message.

Messenger RNA isolation

The medium of neuroblastoma cells growing in log phase was supplemented with 50 μ Ci of [5,6-³H] Uridine for one to two days. Cells were harvested and the cytoplasmic membranes disrupted in 10 mM Tris/ 10 mM NaCl/1% nonidet P-40/10 mM vanaylribonucleoside complexes buffer, pH 7.5. Nuclei were pelleted, the supernatant was collected and buffer added to a final concentration of 0.4M Na acetate/1% SDS/10 mM EDTA at 4°C. This homogenate was extracted with phenol /chloroform/isoamyl alcohol (24:1:1) and RNA was precipitated from the aqueous phase at -20°C by the addition of 2.5 volumes of ethanol. Messenger RNA was

isolated by oligo dT-cellulose chromatography as described in subsequent paragraphs.

Linear 15-30% sucrose gradients were prepared in 0.1 M NaCl/10mM Tris-HCl/1mM EDTA pH 7.5 buffer. Gradients were 10 ml over a 0.3 ml 50% sucrose pad. A 0.3 ml RNA sample in the same buffer was layered on each gradient. Gradients were centrifuged in a Beckman SW-41 rotor at 25,000 rpm at 21-23°C for 16 hours.

The bulk of messenger RNA isolated in this manner from neuroblastoma forms a broad band which peaks at 18S. Two prominent minor peaks, one at 28S and a heavier species, were also present. Although this procedure of isolation proved adequate for isolation of mRNA, it did not yield a preparation which translated in an in vitro system. Therefore another isolation scheme was used.

Approximately 10 x 150 mm plates, containing subconfluent SK-N-SH neuroblastoma were harvested by washing with Dulbecco's buffered saline solution lacking Mg^{++} and Ca^{++} . Cells were washed twice by centrifugation for 1 min. at 150 xg at 4°C. Ten volumes of 6M guanidium thiocyanate/ 5mM sodium citrate/ 0.1 M β -mercaptoethanol and 0.5% N-lauroyl sarcosine buffer pH7.0 were added to the cell pellet and the DNA was sheared by forcing the viscous mass through a hypodermic fitted with an 18G needle. One gram of cesium chloride was added to each 2.5 ml of homogenate. This preparation was then layered on 5.7 M CsCl cushions and centrifuged in a Beckman centrifuge at 35,000 rpm for 17 hours at 20°C in an SW-41 swinging bucket rotor in polyallomer tubes. The RNA containing pellet was dissolved in 10mM Tris/5mM EDTA/1% SDS, pH 7.5 then extracted with an equal volume of chloroform/butanol (4:1). The organic phase was reextracted with buffer and aqueous phases are combined. Then 0.1 volume of 3M sodium acetate and 2.2 volumes of absolute ethanol were added and RNA is precipitated at -20°C overnight. RNA was collected by centrifugation. The RNA pellet was suspended in sterile 20 mM Tris/0.5 M NaCl/ 1mM EDTA pH 7.6 and

applied to an oligo dT-cellulose (Type III, Collaborative Research) chromatography column equilibrated with the same buffer. After extensive washing, the poly (A)⁺ containing mRNA was eluted by washing the column with buffer lacking salt. The mRNA fraction was then brought to 0.5 M with NaCl and passed through an oligo-dT-cellulose column a second time under identical conditions. The yield of poly(A)⁺ mRNA was 1-3% of total RNA.

Total poly(A)⁺ mRNA from SK-N-SH neuroblastoma was translated using a commercial rabbit reticulocyte lysate system (Bethesda Research Laboratories). Messenger RNA was brought up in sterile distilled water. The optimum magnesium concentration was 1mM, and 5 μ Ci of ³H-leucine were added to each reticulocyte reaction mixture, having a total volume of 30 μ l. Translation mixtures were incubated for one hour at 30°C. Reactions were stopped by plunging the mixtures on ice. For assay of acid precipitable counts, 3 μ l aliquots were removed and diluted into 0.5 ml of 3% casamino acids (Difco) in 0.1 N KOH. The mixture was incubated at 65°C for 30 min., then chilled. An equal volume of cold 25% TCA was added and the mixture left standing for 30 min. on ice. This mixture was transferred to GF/A glass fibre discs, washed twice with 5% cold TCA, then with cold ethanol, dried and counted in a scintillation cocktail. As can be seen from figure 1 the incorporation of ³H-leucine into acid precipitable counts is linear with increasing concentration of added poly (A)⁺ mRNA isolated from SK-N-SH neuroblastoma. Concentration of mRNA varied from 0.01 to 1.0 μ g per reaction increasing from left to right. The first two tubes represent endogenous levels.

Polysome Preparation:

Neuroblastoma cells are washed with Dulbecco's phosphate buffer then homogenized with a Potter-Elvehjem homogenizer in 50 mM Tris/25 mM NaCl/5mM MgCl₂/0.25 M sucrose pH 7.5 buffer containing 1 mg/ml bentonite, 0.2 mg/ml

heparin and 1 μ g/ml cycloheximide to make a 20% (v/v) homogenate. The homogenate is centrifuged for 10 min at 25,000 g at 4°C. The supernatant is collected and 0.1 vol of 10% sodium deoxycholate - 10% Triton x-100 is added. Aliquots of this suspension are layered onto a discontinuous sucrose gradient (3 ml of 2.5 M sucrose and 1.5 ml of 2M sucrose in 25 mM Tris/150 mM NaCl/5mM $MgCl_2$ pH7.5 containing 0.2 mg/ml heparin and 1 μ g/ml cycloheximide. Gradients are centrifuged for 17 hours at 20,000 rpm in a Beckman SW-28 rotor. The 2.5 M sucrose layers containing polysomes are pooled and one volume of 2.5 mM Tris/150 mM NaCl/5mM $MgCl_2$ /0.1% Nonidet P-40 pH 7.5 buffer containing cycloheximide and heparin are added. This solution is dialysed against two liters of the same buffer for 20-30 hours. The dialysate is then frozen in a dry ice/ethanol bath and stored at -70°.

Monoclonal Antibody:

The source of anti-acetylcholinesterase antibody is from hybridoma HB-72 (ATCC), a strain produced by Fambrough et al. (1982). HB-72 is maintained in our laboratory in Dulbecco's Modified Eagles medium supplemented with 10% fetal calf serum under 10% CO₂ atmosphere. Hybridoma are grown to 10⁶ cells/ml in exponential phase then transferred to serum free medium for 25-30 hours. Approximately one liter of medium is then collected and centrifuged at 15,000 g for 30 min. at 4°C. The clarified medium was then brought to 50% saturation with ammonium sulfate. This suspension was centrifuged at 15,000 xg for 20 min. at 4°C. The pellet is collected and suspended in 20 mM NaCl/20 mM phosphate buffer pH 7.8 and dialysed against the same buffer for three days at 4°. The dialysate was applied to a 1.7 x 30 cm column of DEAE-DE52 cellulose equilibrated with dialysis buffer. Protein was eluted using a 20 mM to 200 mM NaCl gradient in 20 mM phosphate buffer pH 7.8. Peak fractions were combined and concentrated by ultrafiltration using Amicon XM-50 membrane. The IgG

fraction was adjusted to 50% (v/v) with glycerol, heparin was added to a final concentration of 0.2 mg/ml and the antibody preparation is stored at -20°C.

IgG is tested for intactness by electrophoresis on 7.5% SDS-polyacrylamide gels in the presence and absence of the reducing agent dithiothreitol.

We are currently preparing enough antibody to use in a polysome precipitation.

Preparation of specific mRNA:

The antibody from hybridoma will be used to isolate specific polysomes according to the general scheme of Kraus and Rosenberg (1982). The polysomes prepared as described in the previous section will be thawed, centrifuged to remove aggregates, and suspended in buffer to a concentration of 15 A₂₆₀ units/ml. Polysomes are reacted for 1 hr. at 4°C with purified monoclonal IgG at a ratio of 160 A₂₆₀ units of polysomes per mg. of IgG. Polysome-IgG complexes are immobilized on a protein-A sepharose column equilibrated with 25 mM Tris/150 mM NaCl/5mM MgCl₂/0.1% Nonidet P-40 pH 7.5 buffer. The column is washed thoroughly and ribosomal subunits and specific mRNA are eluted from the column with a buffer containing 25 mM Tris/20mM EDTA pH 7.5.

The acetylcholinesterase mRNA enriched preparation is then purified to isolate poly(A)⁺ mRNA using an oligo-dT cellulose chromatography column. The column will be run without detergent or in the presence of their lithium salts, conditions used successfully in the isolation of total poly (A)⁺ mRNA from SK-N-SH in this laboratory.

The poly(A)⁺ mRNA enriched for acetylcholinesterase message will be used as such in a reticulocyte translation system and radiolabeled polypeptides from in vitro translation will be sized by polyacrylamide gel electrophoresis and characterized by cross reactivity with specific antibody.

We expect shortly to be in a position to use this mRNA preparation as a template for first-strand synthesis using reverse transcriptase and then to make fully double stranded cDNA which will be used in the next phase of this work namely to insert the gene into a cloning vector.

TABLE I. Comparison of Acetylcholinesterase in Neuroblastoma Cell Lines

Assay Conditions: T=30°C, volume 100 μ l, protein content 0.1 mg, activity expressed in nmol/mg protein/hr; passage number in square brackets; at least 2 replicates.

	<u>Expt. 1</u>	<u>Expt. 2</u>	<u>Expt. 3</u>
LAN-1	114[83]	37.4[84]	36.4[84]
LAN-2	84[84]	78.2[85]	73.7[85]
SK-N-SH	101[30]	100.2[30]	83.1[31]
SK-N-MC	13[48]	10 [50]	10.1[50]
IMR-32	20[64]	8.5[65]	5.6[65]

TABLE II. Effect of Inhibitors on Acetylcholinesterase Activity

Values give activity in percent of control. Passage number in square brackets

	BW284c51 2 x 10 ⁻⁵ M	isoOMPA 10 ⁻⁴ M
LAN-1	40 [84]	82 [83]
LAN-2	22.5[85]	76.4[84]
SK-N-SH	4.9[31]	102 [30]
SK-N-MC	35 [50]	90.7[48]
IMR-32	70.6[65]	77.5[64]

Fig. 1

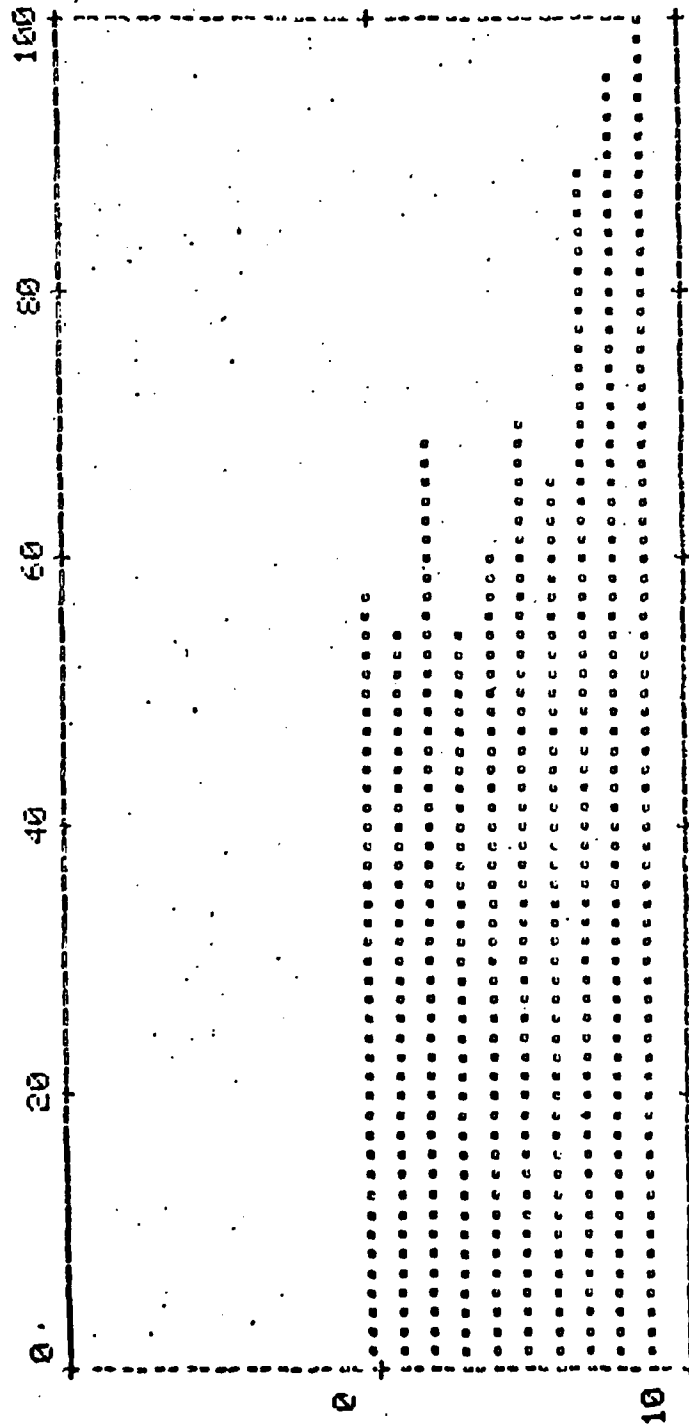
In vitro translation of neuroblastoma mRNA

SINGLE LABEL DIGITAL INTEGRATION

ISOTOPE 1

X AXIS: FRACTIONS

Y AXIS: NORMALIZED ACTIVITY



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